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Continuous decolorization of bleached kraft effluents by *Coriolus versicolor* in the form of pellets

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SUMMARY

Continuous decolorization of kraft black liquor by mycelial pellets of *Coriolus versicolor* in the presence of glucose as co-substrate is discussed. A linear relationship was developed between the rate of decolorization and the liquor concentration. The rate constant was equal to 0.0096 l gmyc^{-1} h^{-1} at 22 °C. During the continuous experiments the pellets exhibited no apparent loss of activity when the liquor concentration was in the range of 400 CU l^{-1} to 5000 CU l^{-1}. However, in the repeated batch experiments a loss of activity was observed which was dependent on the initial liquor concentration. The half-life of pellets was equal to 4.7, 9.4 and 20.2 days for the initial liquor concentration of 1380, 31780 and 6990 CU l^{-1}, respectively. The production of the extracellular enzyme, laccase, was followed but could not be used as an indicator of the ligninolytic activity. The involvement of the intracellular enzymes of *C. versicolor* in the decolorization process is described.

INTRODUCTION

Bleaching operations used in kraft mills generally produce a brown-colored effluent. Most of the color of the effluents is due to the first alkaline extraction (E_1) stage. In addition, the presence of dissolved material released from the chlorination, oxidation and extraction processes has been associated with the dark color of the effluents [33].

Standard aerobic treatment processes such as aerated lagoons or activated sludge which are applied to the effluents for effective reduction of the BOD and COD, cannot efficiently decolorize these effluents. The microorganisms found in the common wastewater treatment systems do not seem to have the capability to degrade lignin and related chromophoric material.

A number of physical and chemical techniques have been used in effluent decolorization with various degrees of success. These techniques have been reviewed by Rush and Shannon [37], Joyce and Petke [16] and Eaton et al. [8]. Precipitation with metal ions or with lime or alum is the most common chemical practice. Physical methods rely on adsorption by activated carbon, ion-exchange resins, membrane separation and extraction. Ozonation, electrodialysis and radiation have also been used in decolorization of wastewaters. Dugal et al. [6] reported that the synergistic action of iron and calcium could cause precipitation of color from the E_1 stage effluent. Eaton et al. [9] also showed that the synergistic interaction of the cations (iron, calcium and aluminum) with the chromophoric material in the E_1 effluent caused an immediate precipitation of chromophoric material. The color was subsequently removed by filtration. In spite of their effectiveness, the high cost of the physical and chemical techniques renders these processes unattractive for industrial applications.

Biological decolorization methods use several classes of microorganisms to degrade the polymeric lignin-derived chromophoric material. Among these classes wood-degrading white-rot fungi have been shown to efficiently and completely degrade and metabolize lignin, resulting in a rapid decolorization of the effluents [7,8,17,28]. The oxidative lignin degradation process requires the presence of co-substrates such as glucose, glycerol, cellobiose or cellulose in order to support fungal growth and lignin metabolism [7,11,20]. The ligninolytic system is induced in response to nitrogen depletion following the primary growth [1,7]. Decolorization of the bleached kraft liquor is believed to be the first industrial application of white-rot fungi [15,23]. The biological color removal is particularly attractive since in addition to color, it also reduces BOD and COD of the effluent.

Eaton et al. [7] reported a 60% color removal of an

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E_1 effluent within 2–4 days by *Phanerochaete chrysosporium*. Later on Eaton et al. [8] and Campbell et al. [2] developed a decolorization process by using a rotating biological contactor (RBC). The treatment system consisted of partially submerged rotating discs over which the mycelium from *Phanerochaete chrysosporium* was adsorbed. The fixed film mycelial color removal (MyCoR) system offered a high surface area per unit volume, low maintenance costs and energy requirements and simple construction and operation. The process could decolorize more than 80% of the E_1 effluent in less than 24 h in the laboratory. Huynk et al. [14] studied the fate of the chlorinated low molecular mass phenols of the E_1 effluent in the MyCoR process. Their results showed that most of the chlorinated phenols and other low molecular weight components of the effluent were removed following the fungal treatment. Marton et al. [31] successfully reduced color from kraft bleach liquor in 3 days with *Coriolus versicolor*. Royer et al. [34] designed and successfully employed a system consisting of fluidized bed reactors with immobilized *C. versicolor* in calcium alginate beads to decolorize the E_1 effluent. Livernoche et al. [27] also used *C. versicolor* and obtained an 80% color removal from bleach plant effluent in 3 days. By immobilizing the same fungus in calcium alginate gel, Livernoche et al. [28] removed 80% of the color of a combined bleach kraft effluent. A 99% color removal from bleach plant effluents within 4 days by *Tinctoporia bornonica* was also reported by Fukuzumi et al. [13].

The use of fungi in mycelial form at high concentrations is difficult because of the problems associated with the recycling of biomass and the non-Newtonian behavior of solutions which result in poor oxygen transfer and poor mixing [19]. An alternative method based on the use of fungi in the form of pellets was described by Cochet [3]. Royer and Rouleau [35] developed a new and simple method for the production of fungal pellets. The method proved to be very efficient for the 26 strains of fungi tested. The technique was successfully employed by Royer et al. [36] for *C. versicolor*. The pellets were used in batch and continuous decolorization of bleached krafts effluents. This paper describes the use of mycelial pellets of *C. versicolor* in continuous decolorization of kraft black liquor. The production of the extracellular enzyme, laccase, and the loss of the pellet activity are also discussed.

MATERIALS AND METHODS

Microorganisms

The basidiomycete *Coriolus versicolor* (Paprican strain no. 52) was used throughout this work. The strain was kept at 4 °C on malt agar slants (Difco).

Effluent

The liquor used in decolorization experiments originated from the first alkaline extraction (E_1) stage effluent of an eastern Canadian kraft mill. In order to obtain a homogeneous substrate and eliminate the influence of dissolved mineral salts, the liquor was ultra-filtered on GS-81 membrane (D.D.S. Nakskon, Denmark, M_w cut-off 10 000) at a pressure of 1 MPa and at a temperature of 55–60 °C before use. The concentrated liquor (560 000 color units (CU) l^{-1}) was diluted to the desired concentration for each individual experiment.

Measurement of color

The color was determined according to the standard CPPA Method [4] as described by Livernoche et al. [28]. The following modifications were made to the method in order to compensate for the variations of pH. The solution was filtered through a Millipore membrane (0.8 μm). The pH and absorbance (465 nm) of the solution were then measured. The absorbance at pH 7.6 was calculated with an empirical equation derived from 65 typical samples:

$$\text{Absorbance } (A;\ \text{pH } 7.6) = 0.108 - 0.023 \times \text{pH solution} \times 1.100 \times \text{Absorbance (pH of original solution)}$$

The following correlation was established between the lignin concentration, the absorbance unit and the unit of color:

$$1\ \text{g}\ l^{-1}\ \text{lignin} = 0.730\ A = 2358\ \text{CU}\ l^{-1}$$

Measurement of laccase activity

The laccase activity was measured according to the method of Leonowicz and Grzywnowicz [26], with the following modifications: acetate buffer 0.1 M was used instead of MES-NaOH buffer, at 30 °C instead of 20 °C.

Preparation of mycelium pellets

Coriolus versicolor was grown on a Petri dish containing malt extract agar for 4 days at 30 °C. Disks of 1 cm in diameter were cut from the peripheral growing zone. Each 500 ml Erlenmeyer containing 200 ml malt extract broth (Difco) was inoculated with four disks. The flasks were shaken for 24 h with a 2.5-cm diameter glass bead to obtain a homogeneous suspension of mycelial particles. This culture was then transferred to another Erlenmeyer without any glass bead and was incubated until pellets of a desired diameter (about 4 mm) were obtained. Culture medium was replaced each day with fresh medium after sedimentation of the mycelial pellets. The pellets were then washed and kept at 4 °C in saline solution (NaCl, 0.85%).

Measurement of glucose concentration

The concentration of glucose in the liquid samples was determined by HPLC as described by Royer et al. [36].

Determination of the loss of pellet activity

A 200-ml batch culture was incubated at 30 °C with vigorous shaking at 250 rpm. Each flask contained approx. 3 g l^{-1} of pellets (based on dry weight). The culture medium consisting of the effluent and glucose was replaced with fresh medium every 48 h. These batch experiments lasted for 18 days with nine renewals of the solution.

Measurement of proteins

The protein content of the microbial pellets was determined by the Folin method [29].

Immobilization of enzymes

1.2 l of *Coriolus versicolor* culture containing approx. 3 g l^{-1} of mycelium (based on dry weight) was spun down for 35 min at 2000 × *g*. The mycelial pellet was washed three times with saline and resuspended in 200 ml sodium acetate buffer (0.1 M) at pH 5. The suspension was grinded and added dropwise to liquid nitrogen. The resulting frozen solid was crushed again and remelted by heat. The cell debris was removed by centrifugation. A 280-ml solution was recovered of which 200 ml was used for enzyme immobilization on a silica support and 80 ml were used in the free-enzyme experiments. The immobilization was performed by the method of Emery et al. [10].

Experimental apparatus

Fig. 1 presents the schematic diagram of the experimental apparatus for the continuous decolorization of bleached kraft effluent. The reactors were built from modified 600-ml Buchner funnels (Pyrex) with porous porcelain bottoms. Air passed through three humidifiers before entering an air distribution chamber from where it was evenly redistributed to four parallel reactors at the rate of 1.5 to 2 volume per volume per min. The reactors were fitted with cylindrical plexiglass tubing to prevent possible overflow of the liquid. A steel screen prevented the wash out of pellets. A chamber at the top of the reactors minimized liquid overflow due to foaming.

Monitoring of experiments

The continuous experiments for the measurement of the rate of decolorization were performed at 22 °C. At

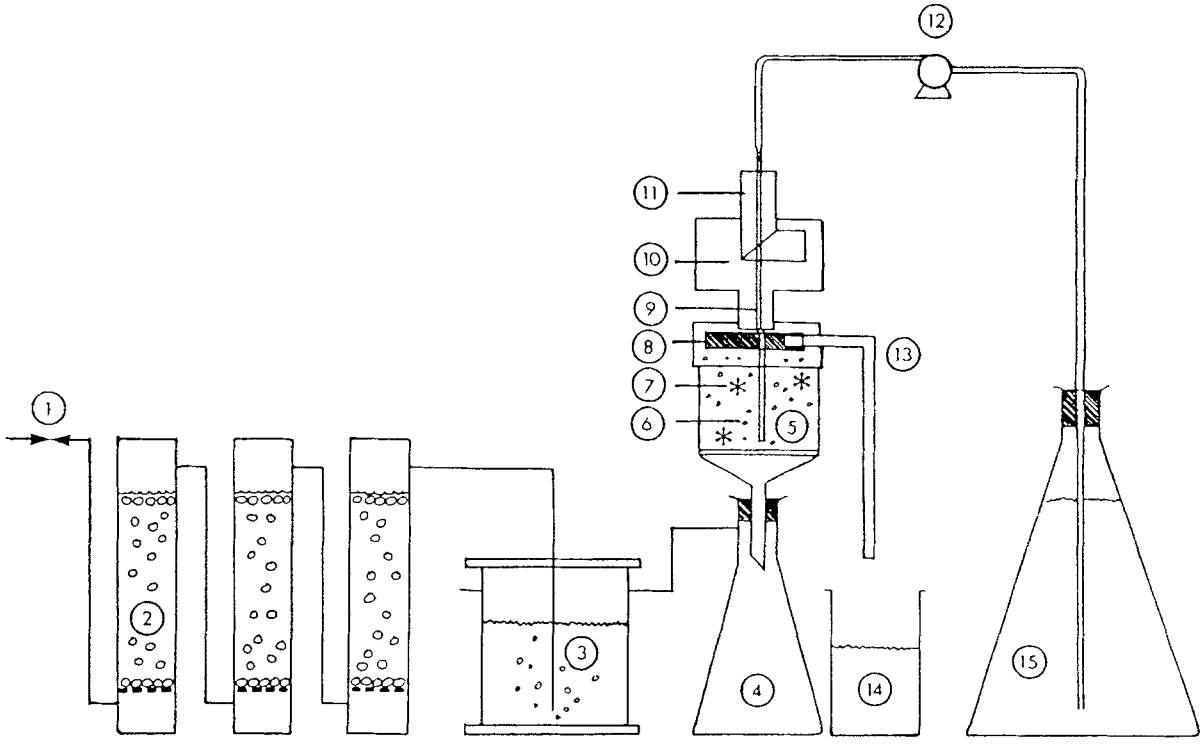

Fig. 1. Schematic diagram of the experimental apparatus for continuous decolorization of bleached kraft effluent. 1, Valve; 2, humidifier; 3, air distributor; 4, Erlenmeyer; 5, reactor; 6, air bubble; 7, *Coriolus versicolor* pellets; 8, outlet with stainless steel screen; 9, air outlet; 10, antifoam system; 11, air outlet; 12, pump; 13, liquid outlet; 14, effluent recovery; 15, liquor to be treated.

time zero, the reactors were filled with demineralized water and a maximum concentration of 5 g l^{-1} fungal pellets were added. The feed liquor was buffered at pH 5 (citrate buffer 0.1 M, 100 ml for 1 l of feed) and 5 g l^{-1} of glucose plus 1 ml l^{-1} of mineral solution [30] were added. The enriched liquor was fed to the reactors through a peristaltic pump. A similar pump was used for the outlet stream. After the first 24 h of experiment, the liquid inside the reactors was withdrawn and filtered through a 0.8-μm millipore filter in order to remove the mycelial particles and to eliminate contamination. Throughout the study, the reactors were operated for eight residence times. Steady state was reached after three to four residence times.

RESULTS AND DISCUSSION

Coriolus versicolor in the form of pellets was used during this work to decolorize kraft liquors. Pellet formation was preferred since it facilitates the retention of the microbial culture inside the reactor and improves the large scale operation of the process. Furthermore, aqueous pellet solutions of concentrations up to 30 g l^{-1} are Newtonian [19]. The recovery and recycling of the pellets are also made easier when compared to filamentous fungi [40]. However, the formation of pellets introduces a number of difficulties such as fragility to mechanical stress and requires air-lift types of mixing for effective operations. The formation of pellets is also often associated with a decrease in the degradation rates of lignin. Kirk et al. [21] showed that under strong agitation, pellet formation with *P. chrysosporium* was favored and the microbial culture could no longer degrade lignin. They also reported [22] that the pellets and not the agitation of the medium were solely responsible for this phenomenon. Campbell et al. [2] used *P. chrysosporium* in the laboratory and showed that the pellet suspension could only slightly decolorize the waste liquors. The same fungus grown on plastic supports decolorized up to 70% of the liquor under similar conditions.

Table 1 presents the results of 20 continuous decolorization experiments. The results indicated that the pellet formation of *Coriolus versicolor* did not inhibit decolorization of the black liquor. The poor activity of the pellets observed by other investigators may be due to poor oxygen transfer efficiencies or to the very large diameter of the pellets used. The pellets used during the present work had a diameter of approx. 4 mm. Large pellets have a higher rate of sedimentation which makes them more attractive from the industrial point of view. Smaller pel-

TABLE 1

Variations of the rates of decolorization, enzyme production and substrate consumption with residence time as well as liquor concentration in continuous culture of *C. versicolor*

Assay No.	Residence time (h)	Pellet diameter (mm)	Liquor concentration (CU l^{-1})	Mean rate of decolorization (CU g myc^{-1} h^{-1})	Mean rate of laccase production (10^4 U laccase g myc^{-1} h^{-1})	Mean rate of glucose consumption (g glucose g myc^{-1} d^{-1})
1	61.2	4.0	1944	8.47	200.00	0.093
2	32.3	5.5	3025	31.52	237.60	0.388
3	31.1	5.5	2688	23.36	73.98	0.139
4	26.8	5.5	2716	22.99	76.32	0.140
5	32.2	5.5	2491	13.93	79.08	0.144
6	23.3	4.1	2573	29.10	48.12	0.105
7	24.1	4.1	3268	37.68	145.14	N.A.
8	19.5	4.1	2515	25.50	15.36	0.101
9	22.8	4.1	2354	15.74	117.90	0.133
10	35.0	3.9	1439	20.07	299.76	0.470
11	33.2	3.9	1203	16.96	143.04	0.315
12	27.1	3.9	1145	14.35	103.26	0.168
13	33.9	3.9	1104	7.22	391.08	0.275
14	35.3	4.8	949	9.25	710.34	0.225
15	33.9	4.8	2012	14.83	450.30	0.230
16	27.1	4.8	2874	32.74	421.80	0.146
17	33.2	4.8	3779	34.80	552.06	0.161
18	34.1	4.3	462	3.19	550.02	0.225
19	26.7	4.3	703	6.96	777.00	0.147
20	33.0	4.3	928	8.15	646.98	0.159

lets may offer a better oxygen transfer and reduction in the formation of hollow centers or lysis zones [32]. However, they are more difficult to form and can cause serious operational problems such as washing out by foam or adherence to screens and plugging. All of the 20 continuous experiments (Table 1) presented stable-steady states as illustrated in Fig. 2. The rate of decolorization was observed to be linearly related to the liquor concentration as presented in Fig. 3. This relationship could be mathematically expressed as:

$$r = k \cdot S$$

where k is the constant of first order reaction rate equation (l g myc^{-1} h^{-1}); r is the specific rate of reaction (CU g myc^{-1} h^{-1}); and S is the concentration of liquor in the reactor (CU l^{-1}). The value of k was determined to be 0.0096 l g myc^{-1} h^{-1} at 22 °C. This relationship is in agreement with the findings of Campbell et al. [2] who observed a correlation between decolorization and color levels. Eaton et al. [8] also demonstrated a first order decolorization of the E_1 effluent by the white-rot fungus *P. chrysosporium*. The rate of decolorization by *C. versicolor* in continuous culture is lower than that in batch culture. In a study of batch and continuous decolorization of bleached kraft effluents by *C. versicolor*, Royer et al. [36] reported that the rate of decolorization was almost ten times higher in batch culture as compared to continuous culture under similar conditions. The rate of decolorization obtained during the present study (Table 1) was lower than that obtained in the MyCoR process [8]. This could be due to the lower temperature of 22 °C used in this work and to the use of pellets with relatively large diameters which could limit the microbial activity as compared to the free mycelium used in the MyCor process.

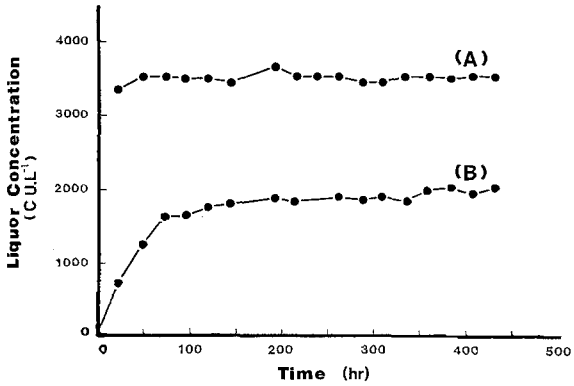

Fig. 2. An example of the color change of bleach kraft liquor in continuous culture of *Coriolus versicolor*. A, color of the liquor entering the reactor; B, color of the liquor leaving the reactor.

Loss of pellet activity

The loss of activity of pellets was determined by following the decrease in the percentage of liquor decolorization in repeated batch cultures. Three different liquor concentrations were used in this assay. As demonstrated in Fig. 4, the percentage of decolorization decreased linearly for all of the liquor samples with different initial concentrations. The loss of activity was most rapid when liquor with the lowest initial concentration of 1380 CU l^{-1} was used. With the two higher liquor concentrations, the rates of decrease in the decolorization were almost equal. It should be noticed that the initial percentage of decolorization decreased with the increase of the liquor concentration. A decolorization of almost 93% was obtained with the liquor concentration of 1380 CU l^{-1} after 24 h.

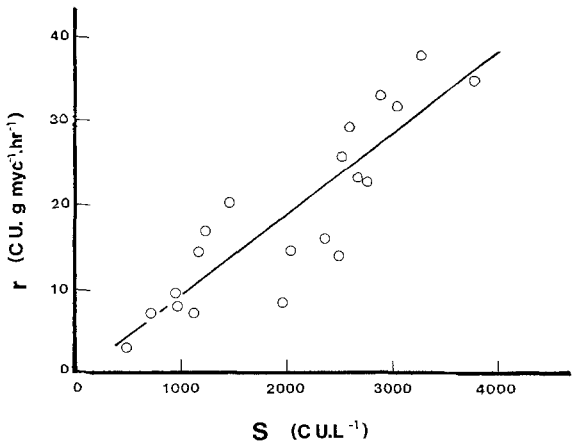

Fig. 3. Rate of decolorization as a function of the liquor concentration in continuous culture of *Coriolus versicolor*. o, experimental values – calculated values.

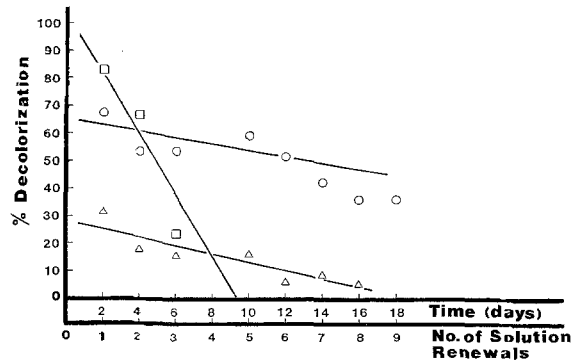

Fig. 4. Loss of activity of pellets of *C. versicolor* in a repeated batch experiment. (□), original liquor concentration, 1380 CU l^{-1}; (○), original liquor concentration, 6990 CU l^{-1}; (△), original liquor concentration, 31780 CU l^{-1}; *T*, 30 °C and mean pellet concentration = 3 g dry weight l^{-1}.

This value decreased to 64% when the initial liquor concentration was 6990 CU l^{-1} and to 28% when the higher concentration of 31 780 CU l^{-1} was used. The half-life of pellets, determined as the time required for their activity to be halved, was dependent on the original liquor concentration (Fig. 4). The half-life was equal to 4.7 days for the liquor concentration of 1380 CU l^{-1}, 9.4 days for the concentration of 31 780 CU l^{-1}, and 20.2 days when the liquor had a concentration of 6990 CU l^{-1}. A possible explanation of the loss of pellet activity is the lack of essential nutrients. Davis et al. [5] showed that in bacterial cultures the addition of manganese and zinc increased the reduction of color and the presence of phosphorous was essential for color removal. The lack of these components could explain the loss of fungal activity observed in the repeated batch experiments. During the 20 continuous experiments, the loss of pellet activity and decolorization were negligible.

Continuous experiments with extreme liquor concentrations

The initial liquor concentration had a remarkable influence on decolorization. At liquor concentrations of less than 400 CU l^{-1} decolorization stopped rapidly and steady-state was not reached. This phenomenon was probably due to microbial contamination which was particularly significant at low liquor concentrations. Microscopic examination of the turbid liquor confirmed the presence of contaminations. On the contrary, when higher liquor concentrations were used, little microbial contamination was observed in spite of non-sterile conditions. This was probably due to the inhibition of contaminating microorganisms by chlorinated chromophores or the generation of toxic metabolites from lignin biodegradation. In these experiments, the liquid inside the bioreactor was filtered after 24 h. Filtration was not necessary during the following days and the liquid remained clear. The residual contamination observed in the 20 non-serial continuous experiments did not seem to affect the decolorization process. A loss of activity was also observed with high substrate concentrations greater than 5000 CU l^{-1} (Table 2). Toxicity of one of the compounds in the kraft liquor was probably responsible for the loss of decolorization activity

Inhibition of the compounds in the bleached kraft effluent was also observed by other investigators. Eaton et al. [7] reported that the ease of fungal decolorization decreased with increasing sodium ion content of the E_1 effluent. Royer et al. [36] also noticed a decrease in the decolorization process due to the presence of non-metabolizable, large molecules in the bleach plant liquor. Therefore, it seems necessary to maintain the liquor concentration in the range of 400 CU l^{-1} to 5000 CU l^{-1} inside the reactor in order to avoid the loss of decolorization activity. The rate equation obtained earlier is valid within this range of liquor concentration.

Enzyme activity

Coriolus versicolor produces an extracellular laccase which plays a role in lignin biodegradation. Table 1 presents the mean rate of laccase production during the continuous experiments. It can be seen that the production of this extracellular enzyme did not follow any specific profile. No correlation could be developed between laccase production and the rate of decolorization. Consequently, laccase production could not be considered as a safe indicator of ligninolytic activity in these experiments. This was probably due to the presence of slight contamination. The contaminants, most of them yeasts and rod-shaped bacteria also exhibited laccase activity. The laccase production by the contaminants measured in batch culture yielded a value of 155–166 units of activity g contaminants^{-1}.

Although the decolorization is primarily due to the action of the extracellular enzymes, there are reports on the involvement of intracellular enzymes in the decolorization process. Watanabe et al. [39] showed that in the presence of a simple sugar and oxygen, sorbose oxidase, an intracellular enzyme of *C. versicolor*, contributed to the

TABLE 2

Loss of the pellet activity at high concentrations of substrate in continuous culture

Assay No.	Residence time (h)	Pellet diameter (mm)	Concentration in reactor (CU l^{-1})	Measurements after four residence times	
				Initial rate (CU g myc^{-1} h^{-1})	Loss h^{-1} (%)
21	29.8	4.7	5403	37.52	0.29
22	28.8	4.7	5342	37.42	0.40
23	28.9	2.2	6511	41.49	0.55
24	29.5	2.2	5926	33.61	0.39
25	20.0	3.0	5779	34.68	0.37
26	22.5	3.0	6042	11.62	0.37

decolorization of the distilleries effluents. In the present study, the cell-free intracellular proteins of *C. versicolor* caused a 20% decolorization of the medium when used in the free form (Table 3). Significant decolorization was obtained when the intracellular compounds in the immobilized form were used (Table 4). Comparison of the results obtained from the control sample with that of the sample containing the inactive immobilized enzyme indicated that an adsorption phenomenon was possibly present in the decolorization process by the intracellular proteins. The chromophores are known to be easily adsorbed on proteins such as hemoglobin. The lower absorbance indicating a positive decolorization obtained with the inactive immobilized enzyme (Table 4) is either due to the adsorption of chromophores on the proteins or to a simple adsorption on the enzyme supports. The adsorption phenomenon also explains the remarkable decolorization obtained with the immobilized enzyme in active state. It is important to note that the contribution of the intracellular enzymes to the actual decolorization process is much less than the extracellular ones.

Utilization of sugar

During this work, glucose was used as a co-substrate. The specific rate of glucose utilization in the continuous cultures ranged from 0.093 to 0.470 g glucose g mycelium^{-1} day^{-1} (Table 1). These values were lower than those necessary for maintenance of the fungal activities as reported by Leisola et al. [25]. The lower values measured in this work could imply that only part of the pellets were active. Another explanation is that the maintenance coefficient data reported in the literature were measured under different conditions such as temperature and the mode of cultivation. It has been shown that the maintenance coefficient is not constant and varies with temperature and growth rate [12]. Ulmer et al. [38] reported that glucose consumption rate in an agitated culture during secondary metabolism was ten times lower than that observed under non-agitated conditions. This indicates that the maintenance coefficient depends on the operating conditions of the culture. The presence of polysaccharides noticed in some of the continuous experiments could also be responsible for the observed lower rate of glucose utilization. In these experiments, the solution was turbid and difficult to filter. Microscopic examination of the medium showed that the turbidity was not caused by microbial contamination. Leisola et al. [24] showed that ligninolytic activity of *P. chrysosporium* was associated with the formation of polysaccharides which, in excess, could be detrimental to oxygen transfer [25]. The formation of these polysaccharides could result in the slow apparent glucose consumption rate since they hydrolyze in acidic conditions and could be consumed as an additional source of carbon.

TABLE 3

Effect of the free intracellular enzymes on decolorization

Condition	Absorbance
Control[a] + 5 ml water	0.736 ± 0.003
Control + 5 ml free-enzyme solution in active state[b]	0.595 ± 0.011
Control + 5 ml free-enzyme solution in inactive state[c]	0.766 ± 0.014

[a] Control consisted of the liquor and glucose solution.

[b] Preparation of the enzyme solution was discussed in the MATERIALS AND METHODS section.

[c] The enzymes were heat inactivated.

TABLE 4

Effect of the immobilized intracellular enzymes on decolorization

Condition	Absorbance
Control + 5 ml water	0.756 ± 0.003
Control + 5 ml immobilized enzyme solution in active state	0.174 ± 0.003
Control + 5 ml immobilized enzyme solution in inactive state	0.581 ± 0.027

Variations of pH

During the continuous experiments, the pH decreased from 0.5 to 1 pH units compared with the pH of the influent. Citrate buffer was consumed very slowly and could represent an alternative to ortho-phthalate and 2,2-dimethylsuccinate [22] or polyacrylic acid [18] buffers used during the study of degradation of lignin by white-rot fungi.

CONCLUSION

The pellets of *Coriolus versicolor* decolorize ultrafiltered kraft liquor in non-sterile conditions with a negligible loss of activity. The process is efficient and the principal limitations originated from the diameter and density of the pellets. An effective decolorization can be obtained in a range of initial concentrations of kraft liquor approx. 400–5000 CU l^{-1}, and in the presence of a simple carbon source such as glucose. In the repeated batch culture, the pellets exhibited a loss of activity dependent on the initial liquor concentration. Laccase production did not reflect the ligninolytic activity of fungal pellets.

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